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Immune Specificity of Murine T-Cell Lines to the Major Outer Membrane Protein of *Chlamydia trachomatis*

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The antigenically variant *Chlamydia trachomatis* major outer membrane protein (MOMP) is a target of antibody-mediated neutralization in vitro, and it is an important protein for designing a subunit vaccine. Knowledge of MOMP T-cell determinants will be essential to elicit rapid and strong immune responses following an encounter with infectious organisms. *C. trachomatis*-specific T-cell lines were derived from MOMP-immunized BALB/c mice and selected with intact organisms. We used these short-term T-cell lines to identify determinants of MOMP that could be recognized by T cells following processing of the intact organism. T-cell line proliferation in response to overlapping MOMP peptides showed that only a peptide encompassing the third variable segment (VS3) elicited a strong proliferative response. We further mapped determinants within the VS3 peptide and found that a sequence-conserved portion of the VS3 peptide elicited T-cell proliferation of T-cell lines from BALB/c mice. Thus, unlike the response to several MOMP peptides with unselected T cells, development of short-term T-cell lines with intact organisms restricted the repertoire of antigens capable of being recognized by MOMP-specific T cells.

Chlamydia trachomatis is an obligate intracellular bacterium that infects the mucosal epithelium of the eye and the genital tract. *C. trachomatis* ocular infections can lead to trachoma, the single most important cause of preventable blindness worldwide (5). Attempts to control trachoma in developing countries by the use of widespread antibiotic therapy have not succeeded (4). In the industrialized world, chlamydial infections of the urogenital tract are a leading cause of sexually transmitted disease. Because these infections are frequently asymptomatic, treatment may not be sought until serious sequelae have developed. The consequences are particularly severe in women, since genital-tract infection can often lead to pelvic inflammatory disease, ectopic pregnancy, or tubal infertility (10).

An effective vaccine would significantly enhance efforts to control both trachoma and the highly prevalent sexually transmitted infections. Because of the immunopathological nature of chlamydial disease, attempts at vaccination with the intact bacterium have led to more severe disease in some individuals, with only limited protection from reinfection in others (7). In human trials, protective immunity is serovar-specific, and numerous animal models have shown that this protective response is T-cell dependent (11). Since there are over 15 serological variants of *C. trachomatis* and because there exists the potential for eliciting immunopathological responses, a subunit approach to vaccine development may be the most useful.

The serovar-specific protective antibody responses are directed at the surface-exposed, major outer membrane protein (MOMP) of *C. trachomatis* (3), and MOMP has thus been the primary focus of vaccine-based research. Sequence analysis of MOMP has revealed that amino acid sequence variation between isolates accounts for the antigenic diver-

sity of this pathogen (13). There are four sequence-variable segments (VS) of the molecule, flanked by five sequence-conserved segments. Monoclonal antibody mapping has determined that complement-independent, neutralizing, type-specific responses are directed at the first and second VS (2, 14, 16).

We recently used recombinant MOMP fusion peptides in an in vivo functional assay to define regions of the molecule capable of eliciting T-cell help for the production of protective antibody (1) and found that with BALB/c mice, a single region of MOMP encompassing the third VS (VS3) is particularly effective in these assays. In the present study, we produced short-term T-cell lines to evaluate MOMP peptides in an in vitro proliferation format. Of the nine recombinant peptides delineating the MOMP protein, only the peptide encompassing VS3 elicited proliferation with chlamydia-specific BALB/c T-cell lines. Further, we identified that a sequence-conserved region of the peptide was stimulatory in these assays. These results demonstrate a marked correlation between in vivo analysis of T-cell help and evaluation by short-term T-cell line proliferation in vitro.

MATERIALS AND METHODS

Growth and purification of *C. trachomatis*. Ocular strains of *C. trachomatis*, B/TW-5/OT and C/TW-3/OT, have been described previously (8). Infectious organisms, elementary bodies (EBs), were grown in McCoy cells, harvested at 72 h, and purified on Renografin gradients as described previously (8). EB preparations were quantitated by Lowry protein determination.

Amplification and cloning of MOMP fragments. The cloning of nine overlapping MOMP gene fragments (CS1, VS1, CS2, VS2, CS3, VS3, CS4, VS4, and CS5 [Fig. 1]) into pGEX has been described previously (1). Smaller VS3 fragments (GG, KE, and AE) were amplified and cloned as

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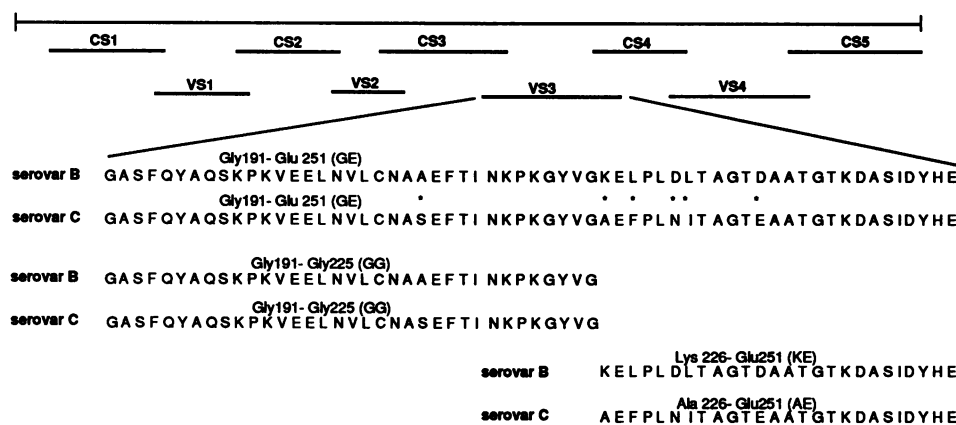


FIG. 1. MOMP peptides expressed as fusion peptides with GST. The nine overlapping peptides derived from the serovar B MOMP sequence are shown and have been described in detail previously (1). The sequence of the VS3 peptide is shown, as well as those of the smaller peptides used for mapping of T-cell recognition sites within VS3. Stars indicate amino acid differences between the serovar B and serovar C sequences. The identities of VS3 peptides used in subsequent figures are in parentheses.

described for the nine MOMP fragments. The following oligonucleotides were used for polymerase chain reaction amplification of peptides GG, KE, and AE: for Gly-191-Gly-225 (GG), 5'-GCA-GGA-TCC-CGA-GCT-TCT-TTC-CAATAT-GC-3' and 5'-AAT-GAA-TTC-CCT-ACA-TA(C/T)-CCT-TTA-GG(C/T)-TT-3'; for Lys-226-Glu-251 (KE), 5'-TAT-GGA-TCC-AAG-GAA-TTG-CCT-CTT-CTT-GAT-CTT-3' and 5'-TTG-GAA-TTC-ATG-GTA-(A/G)TC-AAT-AGA-GGC-3'; and for Ala-226-Glu-251 (AE), 5'-TAT-GGA-TCC-GCG-GAA-TTT-CCA-CTT-AAT-ATT-3' and 5'-TTG-GAA-TTC-ATG-GTA-(A/G)TC-AAT-AGA-GGC-3'. Fusion peptides GG (from serovars B and C), KE (from serovar B), and AE (from serovar C) (Fig. 1) and the glutathione-S-transferase (GST) control were prepared and purified as previously described for nine MOMP fusion peptides (1, 12).

MOMP purification. Purified serovar B or C EBs were boiled in sodium dodecyl sulfate (SDS) sample buffer containing 5% β -mercaptoethanol, and proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE). MOMP was localized by Coomassie brilliant blue staining of a vertical gel strip. The MOMP band was excised from the unstained gel, ground into a fine particulate, and placed in phosphate-buffered saline overnight. The MOMP eluted in this manner was analyzed for purity by SDS-PAGE and quantitated by Lowry protein determination.

Preparation of short-term MOMP-specific T-cell lines. Female BALB/c by mice aged 6 to 8 weeks were obtained from Jackson Laboratories (Bar Harbor, Maine). The mice were immunized by subcutaneous injection with 10 μ g of MOMP from either serovar B or serovar C emulsified in complete Freund's adjuvant. Popliteal lymph nodes were removed from three mice 10 days after immunization, and single-cell suspensions were prepared by filtering through a fine-mesh metal screen. Lymph node cells (4×10^6 /ml) were cultured in 75-cm² flasks in RPMI 1640 with 1 mM 2-ME, 1 μ M glutamine, 2 μ M sodium pyruvate, 100 U of penicillin per ml, 100 μ g of streptomycin per ml, and 0.5% mouse serum at 37°C in an atmosphere of 5% CO₂. Cells derived from mice immunized with serovar B MOMP were cultured with 5 μ g of serovar B EBs or 2 μ g of GST-VS3-B (serovar B sequence) per ml. Cells derived from mice immunized with serovar C MOMP were cultured with 5 μ g of serovar C EBs or 2 μ g of GST-VS3-C (serovar C sequence) per ml. After 4 days of stimulation culture with specific antigen, T cells

(10^5 /ml) were cultured for 7 days with 10^6 accessory cells per ml in the absence of antigen and with 7% rat concanavalin A-stimulated medium and 10% fetal bovine serum instead of mouse serum (rest culture). Viable cells were isolated for assay by Ficoll separation. Fluorescence-activated cell sorter analysis demonstrated that T-cell lines prepared in this manner were >90% CD4⁺. Accessory cells were prepared by irradiation (3,000 rads with ¹³⁷Cs) of single-cell suspensions of syngeneic spleen cells.

T-cell proliferation assay. Ficoll-purified T cells obtained following 11 days of culture, irradiated accessory cells, and antigens were diluted in RPMI 1640 with 1 μ M glutamine, 2 μ M sodium pyruvate, 100 U of penicillin and 100 μ g of streptomycin per ml, and 10% fetal bovine serum. A 100- μ l volume of 10^6 T cells and 10^7 accessory cells per ml were placed in 96-well U-bottom plates. A 100- μ l volume of medium with various concentrations of either fusion protein or purified EBs was added to the wells. Antigens were tested in triplicate wells. After 4 days of incubation, 0.5 μ Ci of tritiated thymidine (³H]TdR) was added to each well and incubated overnight. The cells were harvested onto filters with a cell harvester, and [³H]TdR incorporation was measured in a liquid scintillation counter. The results are expressed as stimulation indices (SI), where SI = counts per minute in test well/counts per minute in medium control well.

RESULTS

In vitro mapping of MOMP T-cell recognition sites. BALB/c mice were immunized with purified MOMP, and short-term T-cell lines were produced by 4 days of stimulation with intact organism followed by 7 days of culture in the absence of antigen. These cells, now predominantly CD4⁺, were used in a T-cell proliferation assay to measure the stimulatory capacity of nine MOMP fusion peptides (Fig. 1). In this assay format, only the peptide encompassing VS3 was stimulatory (Fig. 2). In contrast, we had previously tested the ability of these nine MOMP fusion peptides to elicit proliferation of T cells derived from the spleens of chlamydia-infected mice. In these primary-culture assays, several of the MOMP peptides generated proliferative responses (1). Su et al. (15) also observed several MOMP peptides that were capable of eliciting T-cell proliferation of

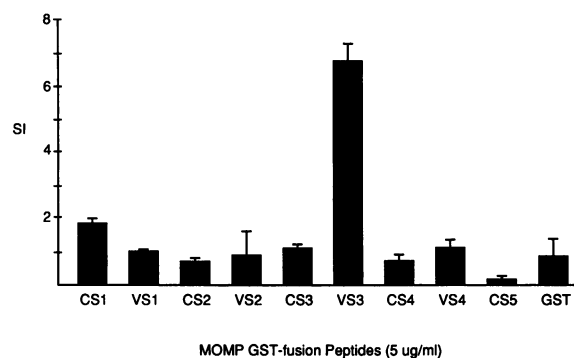


FIG. 2. T-cell proliferation assay with *C. trachomatis*-specific T-cell lines derived from serovar B MOMP-immunized BALB/c mice. Nine GST fusion peptides spanning the MOMP were used as antigens in this assay. GST was used as a control. Proliferation of the T-cell line in response to each antigen was determined by the addition of tritiated TdR on day 4 of culture. Incorporation was measured after an 18-h incubation, and the results were reported as the averages of triplicate wells. SI = counts per minute in antigen wells/counts per minute in medium wells, where SI is the stimulation index. These data are from one of three separate T-cell lines that provided similar results.

primary spleen cells. However, in an in vivo functional assay in which mice are immunized with each of the nine fusion peptides and then challenged with intact organism, the VS3 peptide was significantly more effective in eliciting T-cell help for the production of anti-EB antibodies than the other eight MOMP peptides (1). Therefore, the results with T-cell lines correspond more directly to the in vivo functional data than analysis of primary spleen cell cultures.

Evaluation of VS3 sequence diversity. To evaluate the potential role of sequence diversity in the T-cell response to the GST-VS3 fusion peptide, T-cell lines were developed from mice immunized with either serovar B MOMP or serovar C MOMP and stimulated with the intact organism of the homologous serovar. Proliferative responses of these T-cell lines were then tested with VS3 fusion peptides with amino acid sequences corresponding to either serovar B or serovar C. Serovar B and serovar C were chosen for evaluation, since they are the most sequence divergent and antigenically distinct of the *C. trachomatis* serovariants. As demonstrated in Fig. 3, whether the T cells were selected with serovar B or serovar C antigen, both the VS3-B and the VS3-C peptides stimulated T-cell proliferation. Thus, sequence diversity within the VS3 fragment did not significantly influence T-cell recognition of this peptide in BALB/c mice.

Mapping of T-cell recognition sites within VS3. The assay format with short-term T-cell lines was used to map the T-cell determinants within the large VS3 fusion peptide. Fusion peptides that corresponded to the conserved amino-terminal portion of GST-VS3 and the more variant sequence carboxyl-terminal portion were designed (Fig. 1). The variant-sequence region of the VS3 fragment (peptides KE and AE) was not capable of stimulating EB-selected T cells, whereas the more conserved portion (GG) elicited T-cell proliferation (Fig. 4). However, the smaller GG fragments were not as effective a T-cell antigen as the full-size (GE) GST-VS3 fragment. Optimal recognition may require the larger GE fragment, since reactivity to this peptide may be due to the presence of more than one T-cell determinant, or require processing sites not available in the smaller fusion

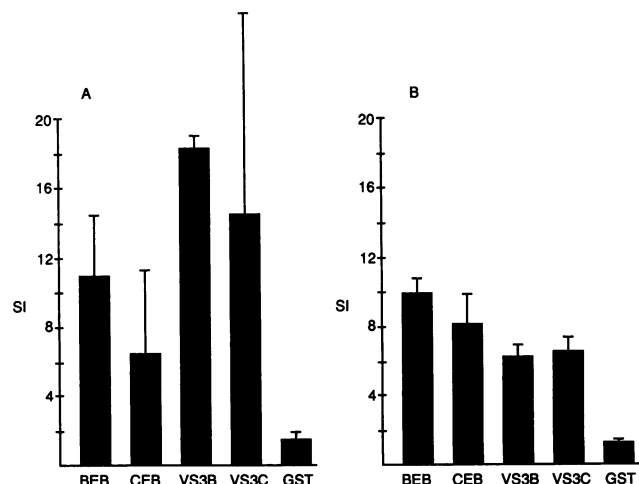


FIG. 3. T-cell proliferation assay with T-cell lines specific for either serovar B or serovar C EBs. These cell lines were tested against GST fusion peptides containing the VS3 sequence (GE) from each serovar to assess the role of sequence diversity in the ability of this MOMP segment to stimulate T-cell proliferation. (A) Serovar B-specific T-cell line; (B) serovar C-specific T-cell line. These data are from one of three separate T-cell lines for datum set A and one of two cell lines for datum set B.

peptides. Analysis of the KE and GG fragments in an in vivo functional assay was consistent with this result; only the conserved GG fragment was capable of priming for a MOMP-specific antibody response in BALB/c mice after challenge with EBs (unpublished observation).

VS3-specific T-cell lines. We also evaluated whether T-cell lines selected with the GST-VS3 fusion peptide could be used for T-cell-determinant mapping as effectively as T cells selected with EBs, since the fusion peptide would not be processed in the same fashion as the native MOMP. T cells

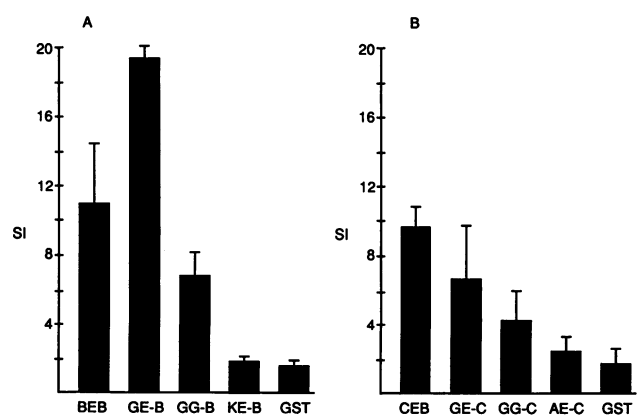


FIG. 4. Mapping of T-cell recognition sites within VS3 with T-cell lines stimulated with serovar B or serovar C EBs. GST fusion proteins containing either all of VS3 (GE), the conserved portion of VS3 (GG), or the variable portions of VS3 (KE and AE) were evaluated for their ability to elicit T-cell proliferation. (A) T-cell line stimulated with serovar B EBs and tested in assay with serovar B EBs, GE (VS3B), GG (serovar B), and KE (serovar B); (B) T-cell line stimulated with serovar C EBs and tested in assay with serovar C EBs, GE (VS3C), GG (serovar C), and AE (serovar C). These data are from one of two separate T-cell lines each for datum sets A and B.

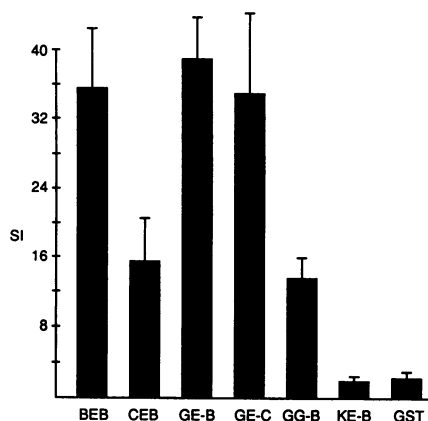


FIG. 5. Mapping of T-cell recognition sites within VS3 with a VS3-B-specific T-cell line. Proliferation of a T-cell line selected by using the GE fusion peptide was tested with serovar B and serovar C EBs and the GE (serovar B), GE (serovar C), GG, and KE fusion peptides. These data are from one of three separate T-cell lines that provided similar results.

derived from MOMP-immunized mice were stimulated in vitro with purified VS3 fusion peptide rather than intact organism. These lines were tested against intact organism and each of the VS3 fragments. The results are shown in Fig. 5. It was significant that T-cell lines selected by using the VS3 fragment proliferated strongly in response to chlamydiae, emphasizing the importance of this region as a T-cell recognition sequence in the context of processing of the entire bacterium. In addition, the observed pattern of reactivity to GST-VS3 and the two fragments (KE and GG) was identical to that seen with lines directed against the intact organism. Thus, fine-mapping studies may be facilitated by using cloned peptides to prepare T-cell lines.

DISCUSSION

The MOMP of *C. trachomatis* is the most promising candidate antigen for designing a subunit vaccine for chlamydia infection. Protection is known to be serovariant specific, and serovar-specific antibodies to MOMP neutralize infectivity in vitro and in vivo (16). An understanding of MOMP T-cell determinants is important, because protective responses are T-cell dependent and a vaccine would require T-cell priming of individuals to generate a rapid and specific immune response following an encounter with the organism. We used short-term T-cell lines to identify MOMP peptides that are recognized by T cells. These were generated by being primed in vivo with purified MOMP and selected in vitro by stimulation with intact organisms. Organisms were used for stimulation to ensure that T-cell determinants recognized following immune processing of the entire organism could be identified.

With BALB/c T-cell lines, the peptide encompassing the VS3 region, including the proximal conserved amino acids, elicited the strongest proliferative response. Whether the T cells were primed, stimulated, or tested with serovariant homologous antigens or serovariant heterologous antigens, T-cell proliferation to the VS3 peptide was observed. These findings suggest that sequence variation within this peptide does not affect recognition by the *H-2^d* haplotype. This was confirmed by using smaller peptides in which the peptide containing the conserved amino acid sequence among sero-

variants was recognized and the peptide containing the variable-sequence portion did not stimulate T-cell proliferation. T-cell proliferative responses to several MOMP peptides have been observed when unselected immune spleen cells are used (1, 15). Significantly, short-term chlamydia-specific T-cell lines in this system restricted the response to the region of MOMP containing VS3. The restricted response of the T-cell lines to one peptide (i.e., VS3) compared with the response in primary cultures to several peptides (1, 15) was a surprising finding, and this suggests that VS3 represents an immunodominant T-cell determinant (9). We have previously found that this peptide, VS3, has the ability to stimulate T cells in vivo that provide help to MOMP-specific B cells (1). Thus, there is a striking correlation between the in vitro proliferation of these lines and previously described in vivo analysis of T-cell help. This correlation between functional assays and in vitro evaluation provides a valuable tool for the analysis of T-cell determinants for vaccine development.

Gajewski et al. (6) showed that different Th subsets proliferate optimally to different antigen-presenting cell populations and, in particular, that Th2 cells proliferate in response to B cells, whereas Th1 cells proliferate optimally to radioresistant cell populations. The predominant antigen-presenting cell in the primary spleen cell cultures is the radioresistant dendritic cell or macrophage with some B-cell contamination of the nylon wool-purified T cells. In the short-term T-cell lines, high numbers of MOMP-specific lymph node-derived B cells are present during the stimulation culture. The presence of a large population of specific B cells may select in vitro for the same population of T helper cells that are effective in vivo for providing T-cell help for the production of antibody. The ability of a particular determinant to be processed and presented effectively by MOMP-specific B cells may be the factor that results in the relationship between the in vitro proliferative response of short-term lines and the in vivo functional analysis. An additional selection factor may occur, because EBs are used during in vitro stimulation and constraints in the processing of native organism-associated MOMP versus denatured MOMP may limit the response to certain peptides.

The importance of VS3 as a T-helper determinant needs to be evaluated by assessment of its function in multiple murine haplotypes and evaluation of sequence diversity recognition in each of these strains. Preliminary results with C3H mice by the in vivo approach demonstrated that these mice recognize the variant-sequence (KE) portion of VS3. However, these assays require large numbers of mice; therefore, the number of variables that can be tested is limited. Analysis of sequence variability in the C3H mouse response and the fine specificity of T-cell determinants with other strains of mice representing a variety of haplotypes should be greatly facilitated by the use of T-cell lines rather than in vivo assays. Thus, the short-term cell lines should facilitate the analysis of multiple murine major histocompatibility complex haplotypes and permit concurrent finer mapping of the functionally important determinants in T-cell recognition. Preliminary data from one experiment with a T-cell line derived from C3H mice showed strong proliferation to the VS3 peptide. This suggests that recognition of T-cell determinants within the VS3 peptide is not restricted only to the *H-2^d* haplotype. If the correlation between the proliferation of short-term lines and functional T-cell help could be demonstrated in humans by using peripheral blood lymphocytes, this would provide a valuable means to analyze the human T-cell response to chlamydia antigens and allow a

safe and relatively noninvasive means to evaluate large numbers of individuals for their capacity to respond to vaccine constructs prior to more-risky studies involving immunization of human subjects.

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